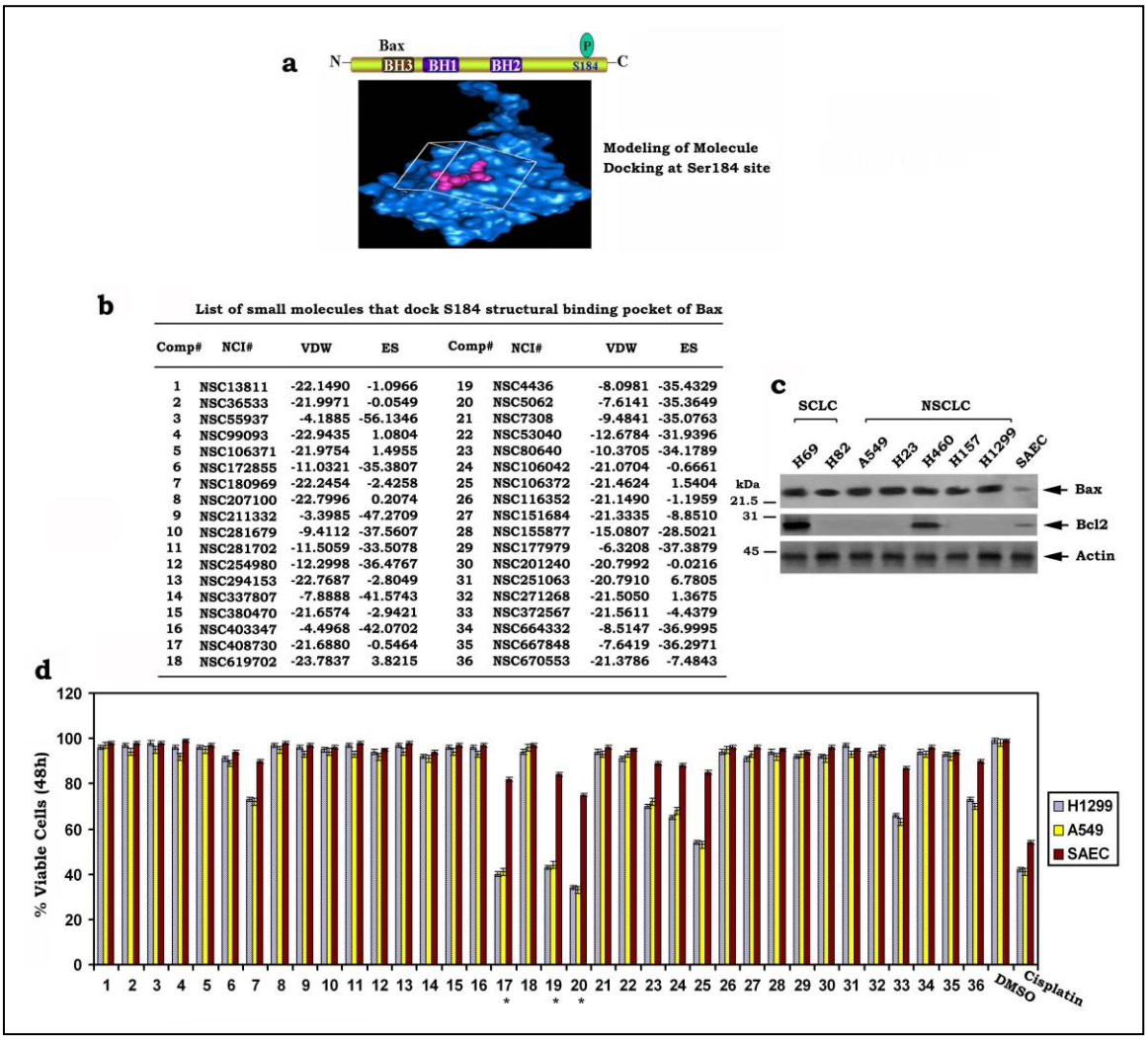
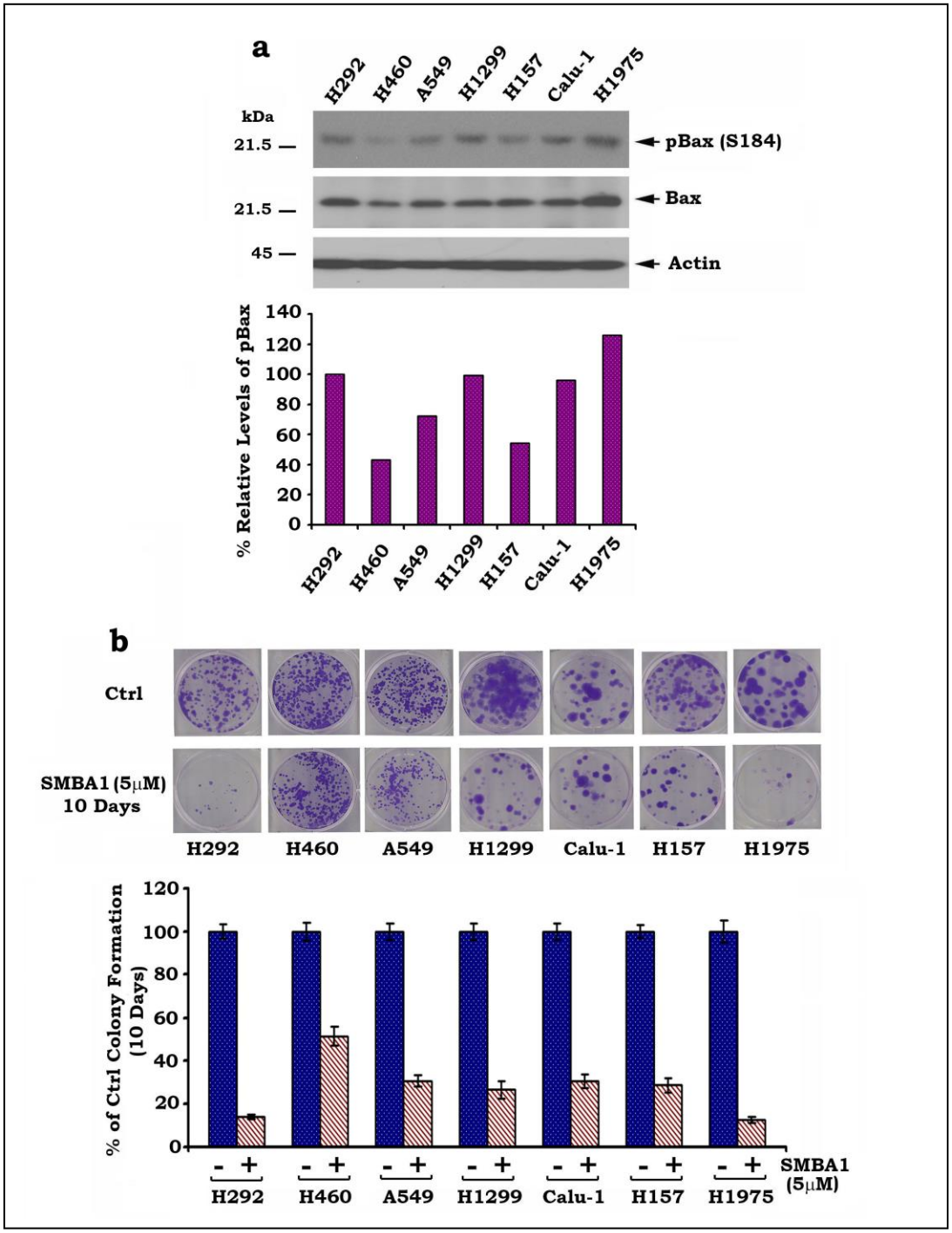
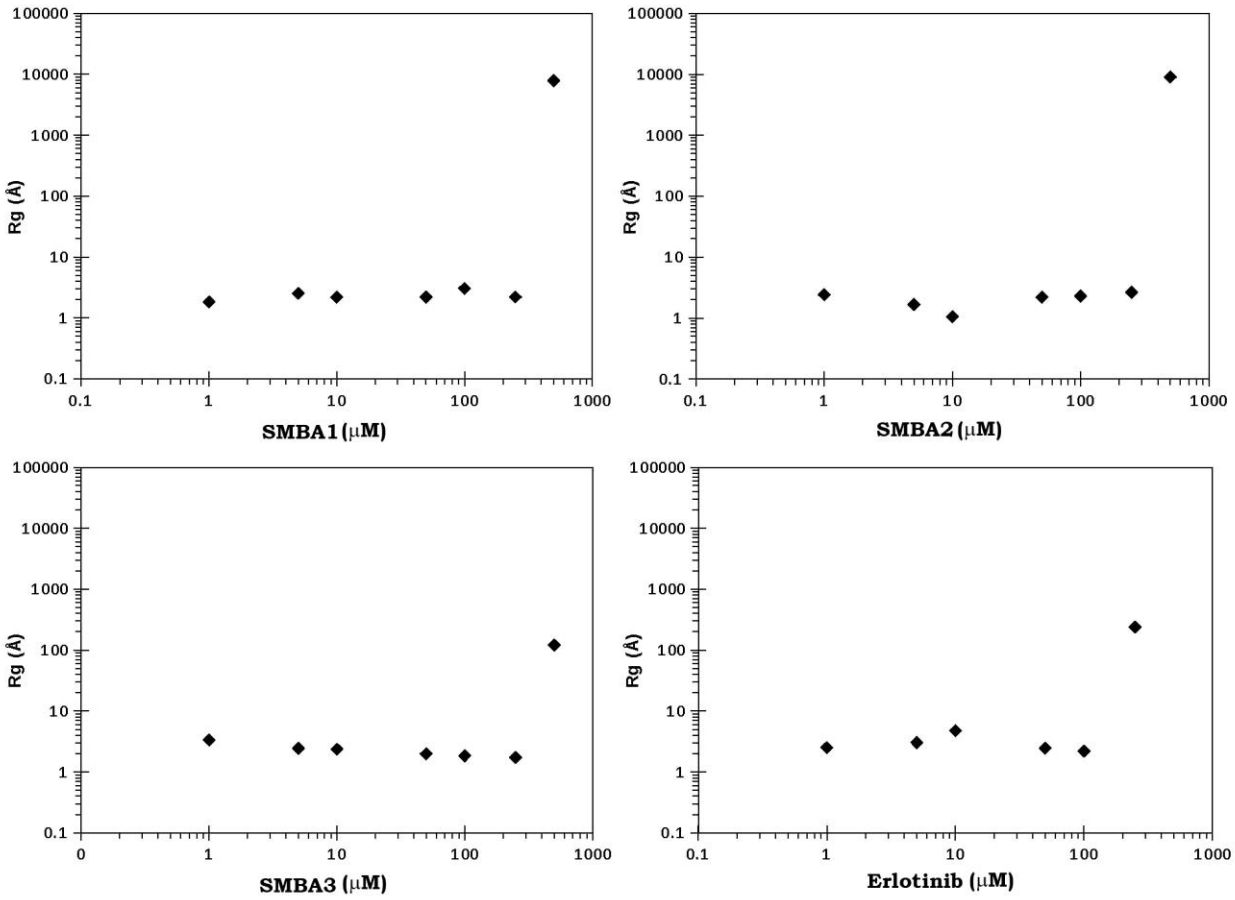


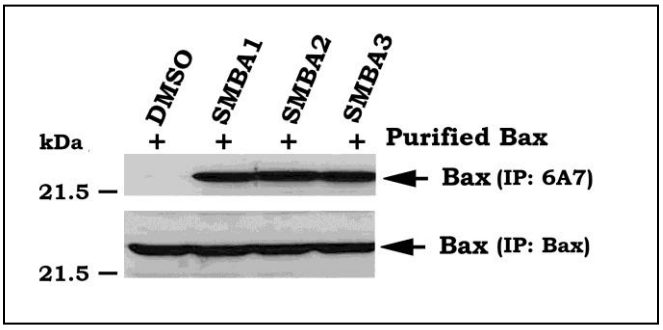
Supplementary Figure 1. Screening of small molecules that structurally target the binding pocket around the S184 site of Bax by the computerized DOCK suite of programs. (**a**) Schematic representation of the Bax homology domains (BH) and the S184 phosphorylation site in Bax protein, and structural modeling of molecule docking around the S184 site. (**b**) The top 36 small molecules that dock the S184 binding pocket of Bax with priority energy scores. (**c**) Expression of Bax or Bcl2 in various lung cancer cell lines or primary normal small airway epithelial cells (SAEC) was analyzed by Western blot. (**d**) H1299, A549 or SAEC cells were treated with the top 36 small molecules (5 μM) for 48h. Cell viability was assessed using ApoAlert Annexin-V kit. DMSO or cisplatin (40 μM) was used as a negative or positive control, respectively. The error bars indicate ± SD.



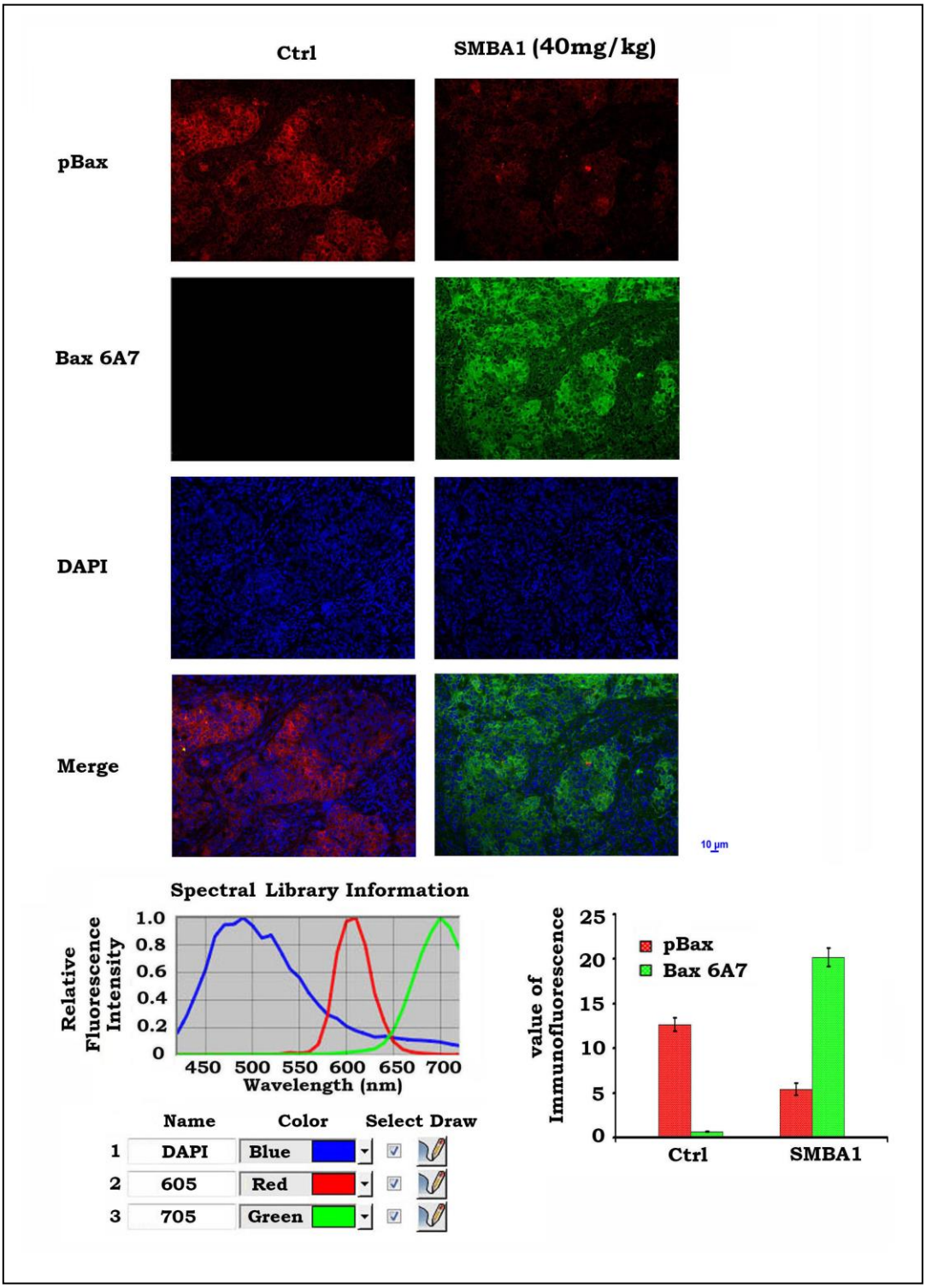
Supplementary Figure 2. Expression levels of pBax and/or total Bax are associated with sensitivity of SMBA1 in various human lung cancer cells. (**a**) Levels of pBax and/or total Bax in a panel of lung cancer cell lines were analyzed by Western blot using phospho-specific S184 Bax or Bax antibodies, respectively. Levels of pBax were quantified using ImageJ software (National Institutes of Health, Bethesda, MD, USA). (**b**) Cell growth was analyzed by colony formation assay following treatment of a panel of lung cancer cell lines with SMBA1 (5μM) for 10 days. The error bars indicate ± SD.



Supplementary Figure 3. Dynamic light scattering (DLS) analysis of SMBA compounds. Various concentrations (1, 5, 10, 50, 100, 250, and 500 μM) of SMBA1, SMBA2, SMBA3 or erlotinib were analyzed by DLS to detect aggregation. Erlotinib, a commonly used lung cancer clinical drug, was used for comparison. The radius of gyration (Rg) of the major component, by mass, was determined using a Dual Integrated Light Scattering system Malvern Zetasizer uV. Except in the case of aggregation, at high concentration and Rg, the major component was 100% of the mass. No self-aggregation of SMBA1, SMBA2 or SMBA3 was observed at concentrations lower than 500 μM. For erlotinib, no self-aggregation was observed at concentrations lower than 250 μM.

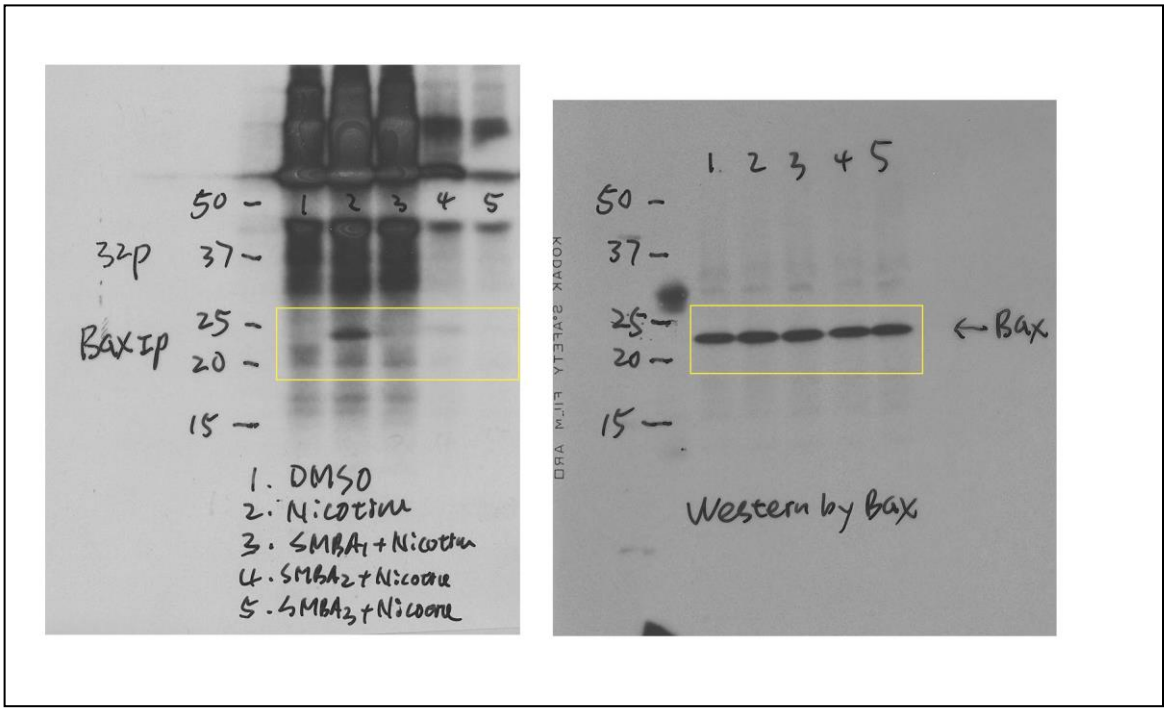


Supplementary Figure 4. SMBA1, 2 and 3 directly induce Bax conformational change in a cell-free system. Purified recombinant Bax protein (10ng/ml) was incubated with SMBA1, SMBA2 or SMBA3 (1 μM) in cold EBC lysis buffer at 4 ^{o}C for 2h. Immunoprecipitation (IP) experiments were performed using 6A7 or Bax antibody, respectively. Bax was analyzed by Western blot using Bax antibody (1:500 dilution).

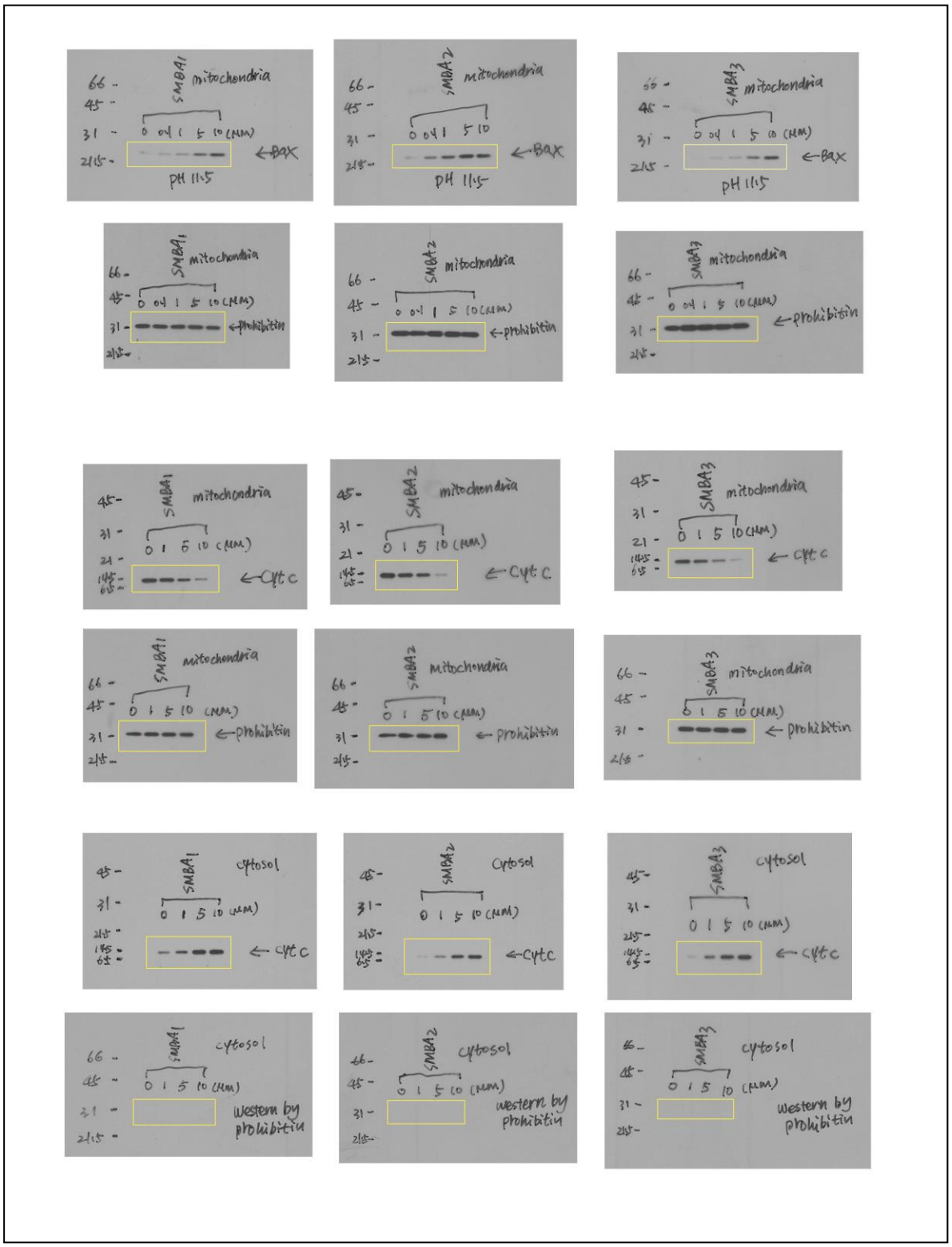


Supplementary Figure 5. **QD-IHF analysis of pBax and 6A7 Bax in tumor tissues**. A549 lung cancer xenografts were treated with vehicle control or SMBA1 (40mg/kg/d) for 10 days.

pBax and 6A7 Bax were analyzed in tumor tissues at the end of experiments by QD-IHF and quantified as described in “Methods”. The error bars indicate ± SD.

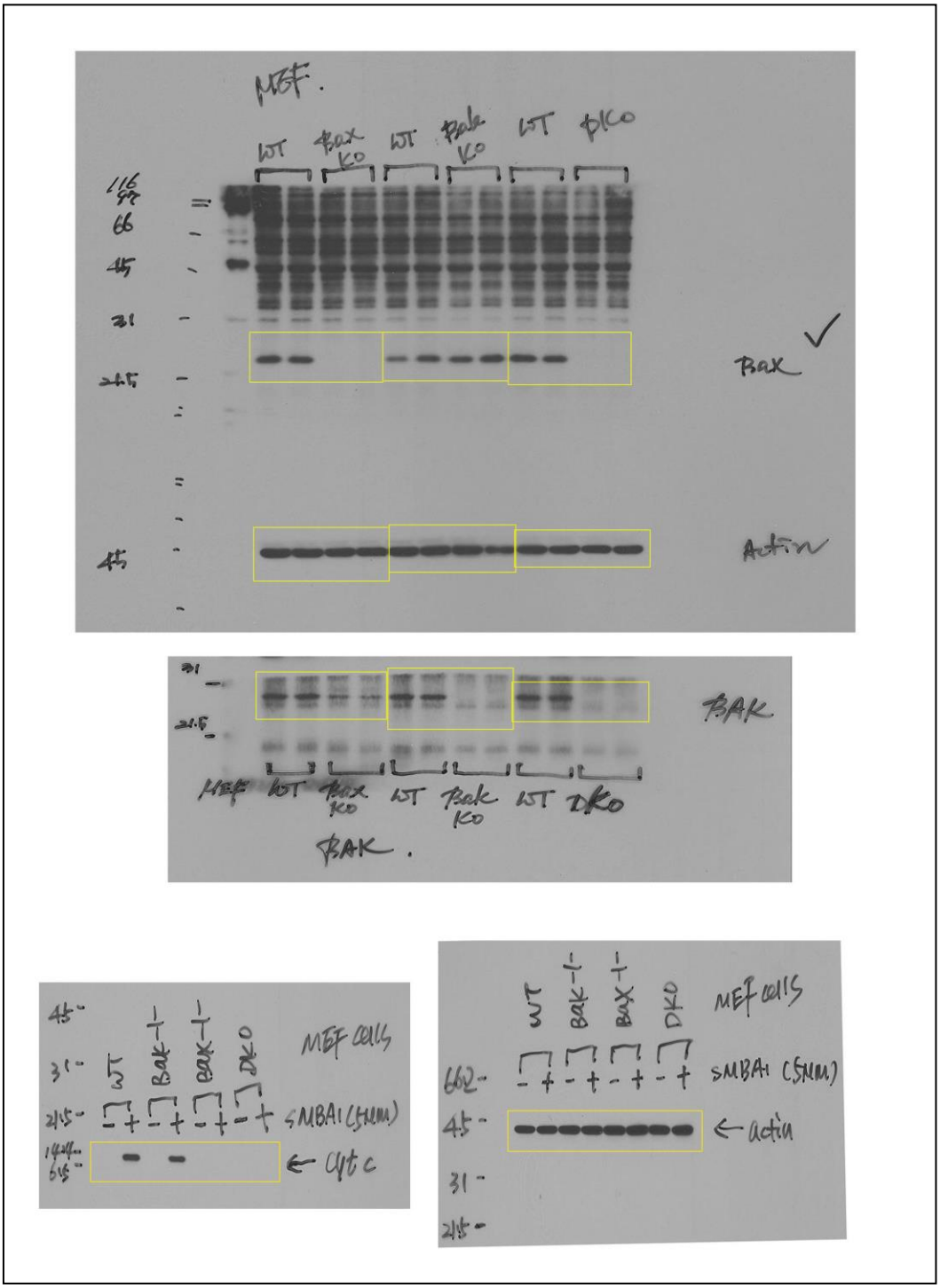


Supplementary Figure 6. (Related to Fig. 2a)
Original autoradiography and Western blots

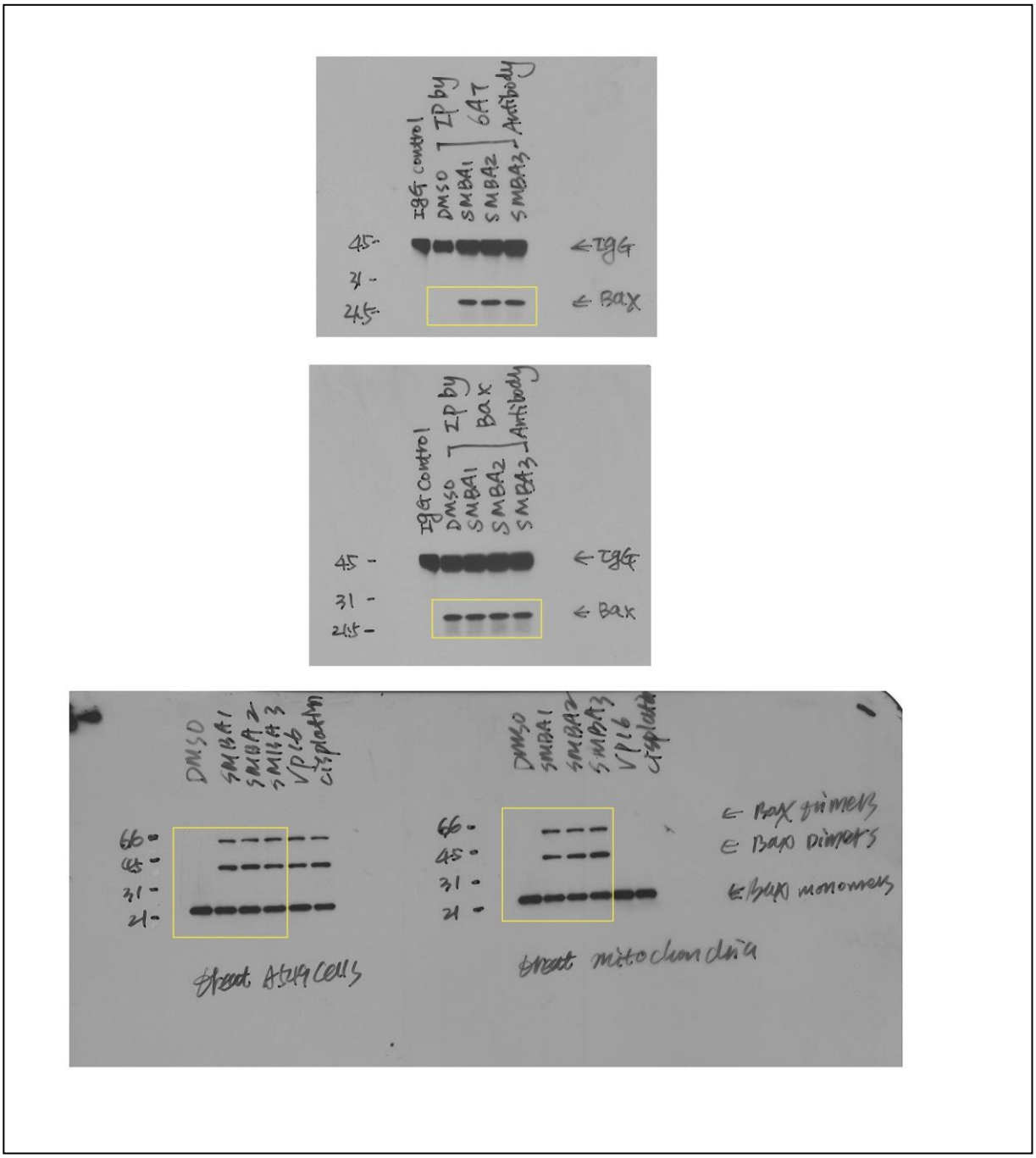


Supplementary Figure 7. (Related to Fig. 3b and c)

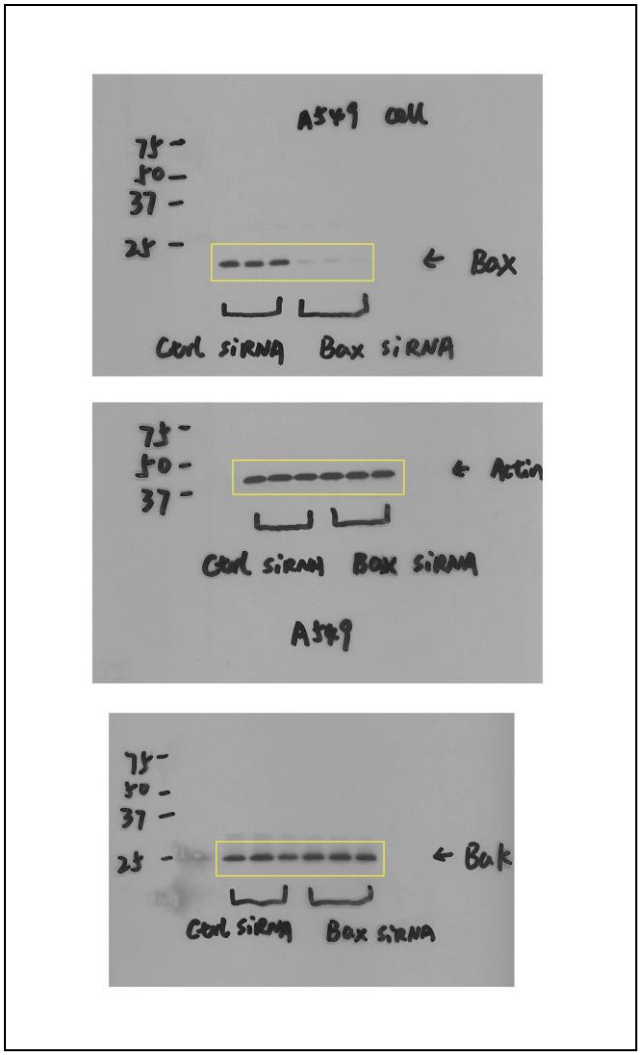
Original Western blots



Supplementary Figure 8 (Related to Fig. 4a and c)
Original Western blots



Supplementary Figure 9 (Related to Fig. 5b, c and d)
Original Western blots



Supplementary Figure 10 (Related to Fig. 8a)
Original Western blots

Supplementary Methods

Dynamic light scattering (DLS) analysis.
DLS analysis for SMBA compounds was performed as described[1, 2, 3]. Briefly, samples of each compound were prepared by first dissolving powdered sample in DMSO. These were then diluted with the same aqueous assay buffer (pH 7.4) used above, to yield serial dilutions of 1, 5, 10, 50, 100, 250, and 500 µM. For each sample concentration, the radius of gyration (Rg), of the major component by mass, was determined using 4µl aliquots pipetted into a quartz sample cuvette placed in a Malvern Zetasizer µV Dynamic Light Scattering (DLS) instrument (Malvern Instruments Ltd, Malvern, UK) at 25°C [1, 2]. The major component was always 100% of the mass except for when the sample displayed a large Rg and had begun to aggregate. In the absence of aggregation the instrument's automatic intensity optimization routine always used the same settings: 100%. As recommended by the manufacturer, the autocorrelation curves were examined to ensure that scattering from low sample concentrations was not too small to distinguish from buffer.

Fluorescence polarization assay. The fluorescent Bak BH3 peptide (3 nM) was incubated with purified recombinant Bax protein (6 nM) in the absence or presence of increasing concentrations (*i.e*. 0~500 nM) of SMBA (s) in a 96-well assay plate. Polarization, defined as millipolarization units (mP), was measured at room temperature with a fluorescence microplate reader (Wallace, CA). A negative control (DMSO, 3 nM peptide and assay buffer) and a positive control (DMSO, 3 nM peptide, 6 nM Bax protein and assay buffer) were used to determine the range of the assay. The percentage of inhibition was determined by (1-[(mP value of well-negative control)/range)] × 100%. The inhibitory constant (K_i) value was calculated using Microsoft Excel as described [4]. Values are the mean ± S.D. for three separate experiments run in duplicate.

Metabolic labeling, immunoprecipitation, and Western blot analysis. Cells were washed with phosphate-free RPMI medium and metabolically labeled with [^{32}P]orthophosphoric acid for 60 min. After nicotine or SMBA addition, cells were washed with ice-cold 1× PBS and lysed in ice-cold EBC buffer (0.5% Nonidet P-40, 50mM Tris, pH 7.6, 120mM NaCl, 1mM EDTA, and 1mM-β-mercaptoethanol) containing protease inhibitor cocktail set I. Bax was immunoprecipitated using an agarose-conjugated Bax antibody. The samples were subjected to 12% SDS-PAGE, transferred to a nitrocellulose membrane, and exposed to Kodak X-Omat film at -80 °C. Bax phosphorylation was determined by autoradiography. The same filter was then probed by Western blot analysis using a Bax antibody and developed using an ECL Kit from Amersham Biosciences as described previously [5].

Immunofluorescence
A549 cells were cultured on a Lab-Tek® chamber slide (Nalge Nunc International) and treated with SMBA. Cells were washed with 1× PBS, fixed with ice-cold methanol/acetone (1:1) for 5 min, and blocked with 10% goat serum for 20 minutes. Then, cells were incubated with rabbit against human Bax (1:100) and mouse against human Cyt c (1:100) primary antibodies for 60 min. After washing, samples were incubated with fluorescein isothiocyanate-conjugated anti-rabbit and rhodamine-conjugated anti-mouse secondary antibodies (1:200 dilution) for 60 min. Cells were washed with 1× PBS, the slides were sealed in mounting medium and observed

under a fluorescent microscope (Zeiss). Pictures were taken and colored with the same exposure setting for each experiment. For mitochondrial staining, living cells were incubated with 20 ng/ml mitotrack for 30 minutes, then fixed and blocked as previously described. To determine subcellular regions of protein co-localization, individual red- and green-stained images derived from the same field were merged using Openlab 3.1.5 software from Improvision, Inc. (Lexington, MA). Areas in *yellow* indicate the protein co-localization.

Subcellular fractionation

Cells (2×10^7) were washed with cold 1× PBS and resuspended in isotonic mitochondrial buffer (210 mM mannitol, 70 mM sucrose, 1 mM EGTA, 10 mM Hepes, pH 7.5) containing 10% protease inhibitor mixture set I, homogenized with a polytron homogenizer, and then centrifuged at 1000 × *g* for 5 min to remove the nuclei and unbroken cells. The supernatant was centrifuged at 13,000 × *g* for 10 min to pellet mitochondria as described previously [6]. The resulting supernatant is the cytosolic fraction. Mitochondria were washed twice with mitochondrial buffer and resuspended in 1% Nonidet P-40 lysis buffer, rocked for 60 min, and then centrifuged at 17,530 × *g* for 10 min at 4 °C. The resulting supernatant containing mitochondrial proteins was collected. Protein (50 µg) from each fraction was subjected to SDS-PAGE. Cyt c was analyzed by Western blot. The purity of fractions was confirmed by assessing localization of the mitochondria-specific protein, prohibitin [7].

Bax insertion assay

A549 cells were treated with SMBA(s), mitochondria were isolated by subcellular fractionation, resuspended in freshly prepared 0.1 m Na_2CO_3, pH 11.5, and incubated on ice for 30 min. The samples were then centrifuged at 200,000 × *g* for 30 min, and the alkali-extracted membrane pellet was resuspended with 1% Nonidet P-40 lysis buffer, rocked for 60 min, and then centrifuged at 17,530 × *g* for 10 min at 4 °C. The supernatant containing the nonextractable mitochondrial proteins was collected and subjected to SDS-PAGE. The alkali-resistant Bax (*i.e.* nonextractable or integral) was determined by Western blot using a Bax antibody (1:500) as described previously [8].

Colony formation assay

Cells were plated in 6-well plates at a density of 500 per well. The next day, cells were treated with SMBA(s). The medium was replaced with fresh medium containing the corresponding concentration of the compounds every 3 days. After 10 days of treatment, the medium was removed and cell colonies were stained with 0.1% crystal violet in 20% methanol and counted as described previously [9].

Cell viability assay. Apoptotic and viable cells were detected using a PI- Annexin-V kit (Clontech, Mountain View, CA) according to the manufacturer's instructions. The percentage of viable cells or apoptotic cells was determined by fluorescence-activated cell sorter analysis as described previously [6, 10]. Cell viability was also confirmed using the trypan blue dye exclusion method [10]

Immunohistochemistry (IHC) analysis

After treatment of mice with SMBA, tumors were harvested, fixed in formalin and embedded in paraffin. Representative sections from paraffin-embedded tumor tissues were analyzed by IHC

staining using an active caspase 3-specific antibody (1:100 dilution). Active caspase-positive cells in tumor tissues were scored at 400 × magnification. The average number of positive cells per 0.0625 mm^2 area was determined from three separate fields in each of three independent tumor samples as described [11].

Quantum Dot-based immunohistofluorescence (QD-IHF) and quantification. QD-IHF for measurement of pBax and 6A7 Bax in tumor tissues was performed as described previously [12, 13, 14]. Briefly, harvested tumors were embedded in paraffin and cut into 4-μm sections. After de-paraffinization and rehydration, antigen retrieval was performed by heating with citric acid (10 mmol/L, pH 6.0) in a microwave for 10 min. The tissue slides were blocked with 2.5% normal horse serum for 10 min before the primary antibody incubation. Rabbit anti-human pBax and mouse anti-human 6A7 Bax antibodies were mixed at 1:50 dilution in 1×PBS containing 2.5% horse serum. Normal rabbit IgG was used as a negative control. Tissue sections were incubated with a mixed solution of pBax (1:50) and 6A7 (1:50) antibodies overnight at 4°C. After washing with 1×PBS three times, QD605 goat anti-rabbit IgG conjugate (red) and QD705 goat anti-mouse IgG conjugate (green) secondary antibodies at 1:50 dilution (Invitrogen Life Technologies Inc., Carlsbad, CA) were added to the slides with further incubation for 1h at 37°C. The slides were washed three times with 1×PBS, counterstained with DAPI, mounted and stored at 4^{o}C under dark conditions. QD imaging and quantification procedures were performed as described previously[14]. The Nuance™ fluorescence microscope system (CRi consolidated with Caliper, a PerkinElmer company, Hopkinton, MA) was used for quantification of the QD-IHF signals. All cubed image files were collected from tumor tissue slides at 10 nm wavelength intervals from 420-720 nm, with an auto exposure time per wavelength interval at 200~400x magnification. Taking the cube with a long wavelength band pass filter allowed transmission of all emission wavelengths above 420 nm. Both separated and combined QD images were obtained after establishing the QD spectral library and unmixing the image cube. For each tissue slide, 10 cubes were taken. The background signal was subtracted for accurate quantification of the QD signals. The average of each QD signal was obtained by selecting tumor areas on each cube for quantification by Nuance imaging software (Caliper/PerkinElmer). An average reading from the 10 cubes was obtained as a total average signal count of each tissue slide for both QD signals.

Vector-based gene silencing of *Bax*. Bax shRNA and pSilencer™ 2.1-U6 hygromycin plasmids were obtained from Ambion (Austin, TX). The *Bax* DNA target sequence for siRNA design is AACTGATCAGAACCATCATGG. Bax shRNA was cloned into the pSilencer™ 2.1-U6 hygromycin plasmids as described previously[5]. The pSilencer™ 2.1-U6 hygromycin plasmids bearing Bax hairpin siRNA were transfected into A549 cells using Lipofectamine™ 2000 according to the manufacturer's instructions. The stable clones persistently producing Bax siRNA were selected using hygromycin (0.8 mg/ml). The levels of Bax expression were analyzed by Western blot using a Bax antibody (1:500 dilution).

Mouse blood analysis. Whole blood (250μL) was collected in EDTA-coated tubes via cardiac puncture of anesthetized mice for hematology studies. Specimens were analyzed for white blood cells (WBC), red blood cells (RBC), platelets (PLT), alanine aminotransferase (ALT), aspartate aminotransferase (AST) and blood urea nitrogen (BUN) in the Clinical Pathology Laboratory at the University of Georgia (Athens, GA).

Supplementary References

1. Frenkel YV, Clark AD, Jr., Das K, Wang YH, Lewi PJ, Janssen PA*, et al.* Concentration and pH dependent aggregation of hydrophobic drug molecules and relevance to oral bioavailability. *J Med Chem* 2005, **48**(6)**:** 1974-1983.

2. Pohjala L, Tammela P. Aggregating behavior of phenolic compounds--a source of false bioassay results? *Molecules* 2012, **17**(9)**:** 10774-10790.

3. LaPlante SR, Carson R, Gillard J, Aubry N, Coulombe R, Bordeleau S*, et al.* Compound aggregation in drug discovery: implementing a practical NMR assay for medicinal chemists. *J Med Chem* 2013, **56**(12)**:** 5142-5150.

4. Bruncko M, Oost TK, Belli BA, Ding H, Joseph MK, Kunzer A*, et al.* Studies leading to potent, dual inhibitors of Bcl-2 and Bcl-xL. *J Med Chem* 2007, **50**(4)**:** 641-662.

5. Xin M, Deng X. Nicotine inactivation of the proapoptotic function of Bax through phosphorylation. *J Biol Chem* 2005, **280**(11)**:** 10781-10789.

6. Deng X, Gao F, Flagg T, Anderson J, May WS. Bcl2's flexible loop domain regulates p53 binding and survival. *Mol Cell Biol* 2006, **26**(12)**:** 4421-4434.

7. Ikonen E, Fiedler K, Parton RG, Simons K. Prohibitin, an antiproliferative protein, is localized to mitochondria. *FEBS Lett* 1995, **358**(3)**:** 273-277.

8. Xin M, Deng X. Protein phosphatase 2A enhances the proapoptotic function of Bax through dephosphorylation. *J Biol Chem* 2006, **281**(27)**:** 18859-18867.

9. Liu Y, Sun SY, Owonikoko TK, Sica GL, Curran WJ, Khuri FR*, et al.* Rapamycin induces Bad phosphorylation in association with its resistance to human lung cancer cells. *Mol Cancer Ther* 2012, **11**(1)**:** 45-56.

10. Deng X, Xiao L, Lang W, Gao F, Ruvolo P, May WS, Jr. Novel role for JNK as a stress-activated Bcl2 kinase. *J Biol Chem* 2001, **276**(26)**:** 23681-23688.

11. Oltersdorf T, Elmore SW, Shoemaker AR, Armstrong RC, Augeri DJ, Belli BA*, et al.* An inhibitor of Bcl-2 family proteins induces regression of solid tumours. *Nature* 2005, **435**(7042)**:** 677-681.

12. Xing Y, Chaudry Q, Shen C, Kong KY, Zhau HE, Chung LW*, et al.* Bioconjugated quantum dots for multiplexed and quantitative immunohistochemistry. *Nat Protoc* 2007, **2**(5)**:** 1152-1165.

13. Xu J, Muller S, Nannapaneni S, Pan L, Wang Y, Peng X*, et al.* Comparison of quantum dot technology with conventional immunohistochemistry in examining aldehyde

dehydrogenase 1A1 as a potential biomarker for lymph node metastasis of head and neck cancer. *Eur J Cancer* 2012.

14. Huang DH, Su L, Peng XH, Zhang H, Khuri FR, Shin DM*, et al.* Quantum dot-based quantification revealed differences in subcellular localization of EGFR and E-cadherin between EGFR-TKI sensitive and insensitive cancer cells. *Nanotechnology* 2009, **20**(22)**:** 225102.